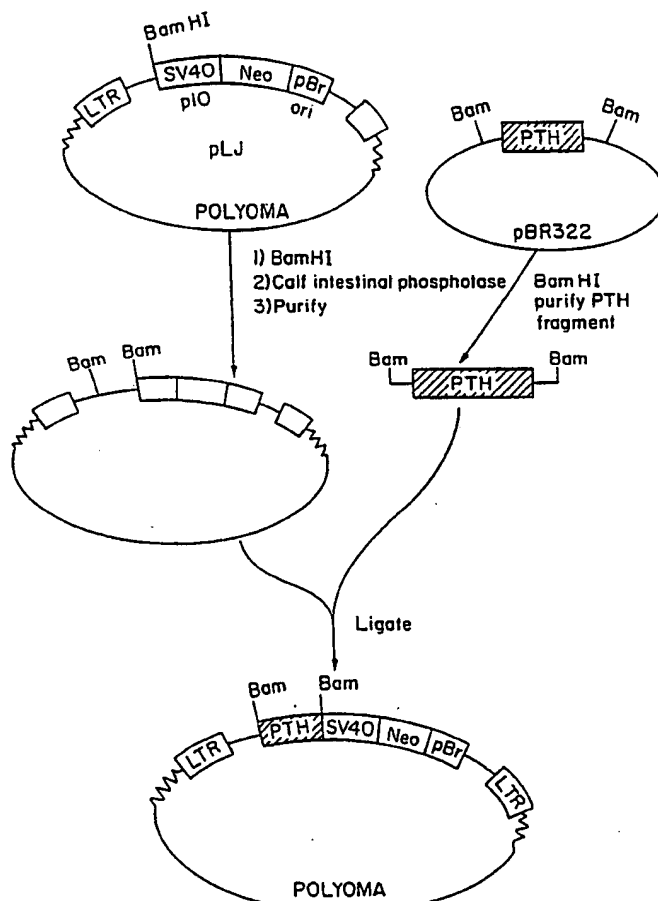# PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 5/00, 15/00, A61L 27/00 A61L 33/00	**A1**	(11) International Publication Number: **WO 89/ 05345** (43) International Publication Date: 15 June 1989 (15.06.89)

(21) International Application Number: PCT/US88/04383

(22) International Filing Date: 8 December 1988 (08.12.88)

(31) Priority Application Number: 131,926

(32) Priority Date: 11 December 1987 (11.12.87)

(33) Priority Country: US

(71) Applicants: WHITEHEAD INSTITUTE FOR BIOM-EDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). HOWARD HUGHES MEDICAL INSTITUTE [US/US]; 6701 Rockledge Drive, Bethesda, MD 20817 (US).

(72) Inventors: WILSON, James, M. ; 3686 River Pines Drive, Ann Arbor, MI 48103 (US). MULLIGAN, Richard, C. ; 441 Franklin Street, Cambridge, MA 02138 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETIC MODIFICATION OF ENDOTHELIAL CELLS

(57) **Abstract**

Endothelial cells transduced with genetic material encoding a polypeptide or protein of interest and, optionnally, a selectable marker, as well as methods for making and using the transduced endothelial cells. Such endothelial cells are useful in improving the performance of vascular grafts and in delivering the encoded polypeptide or protein, such as an enzyme, a hormone or a drug, to an individual who has had a graft or implant of the transduced cells.

GENETIC MODIFICATION OF ENDOTHELIAL CELLS

Description

Background

The endothelium is a single layer of flattened
05 transparent cells which are joined edge to edge, with the
result that they form a membrane of cells. Endothelial
cells originate during development from the embryonic
mesoblast or mesoderm. They occur on the free surfaces
of serous membranes, in the anterior chamber of the eye
10 and on the surface of the brain and spinal cord. In
addition, they form the lining membrane of the heart,
blood vessels and lymphatics.

It is possible, using methods developed in recent
years, to attain interspecies genetic recombination.
15 Genes derived from different biological classes are able
to replicate and be expressed in a selected micro-
organism. Therefore, it is possible to introduce into a
microorganism genes specifying a metabolic or synthetic
function (e.g., hormone synthesis, protein synthesis,
20 nitrogen fixation) which is characteristic of other
classes of organisms by linking the genes to a particular
viral or plasmid replicon.

Since the late 1970s, progress has been made toward
the development of general methods for introducing cloned
25 DNA sequences into mammalian cells. At the present time,

-2-

however, there is a need for an effective method of
stably introducing selected genetic material of interest
into endothelial cells and enabling them to express it,
thus producing the encoded protein or polypeptide.

05 Summary of the Invention

The invention described herein relates to
genetically engineered endothelial cells and particularly
to genetically engineered endothelial cells which express
selected genetic material of interest (DNA or RNA) which
10 has been incorporated into them, for example by means of
a retroviral vector having a recombinant genome which
includes the genetic material of interest. It also
relates to methods of stably introducing into endothelial
cells such genetic material and methods of using the
15 genetically engineered endothelial cells.

Endothelial cells of this invention have stably
incorporated in them genetic material of interest, which
encodes a product (e.g., a protein or polypeptide) whose
production in the endothelial cells is desired. The
20 modified endothelial cells express the incorporated
genetic material (produce the encoded product). This
genetic material of interest is referred to herein as
incorporated genetic material. The incorporated genetic
material can be any selected DNA of interest (e.g., all
25 or a portion of a gene endocing a product of interest) or
RNA of interest. It can be, for example, DNA or RNA
which is present in and expressed by normal endothelial
cells; DNA or RNA which does not normally occur in
endothelial cells; DNA or RNA which normally occurs in
30 endothelial cells but is not expressed in them at levels

which are biologically significant (i.e., levels sufficient to produce the normal physiological effects of the protein or the polypeptide it encodes); DNA or RNA which occurs in endothelial cells and has been modified
05 so that it is expressed in endothelial cells; and any DNA or RNA which can be modified to be expressed in endothelial cells, alone or in any combination thereof. Endothelial cells of the present invention can also express genetic material encoding a selectable marker,
10 thus providing a means by which cells expressing the incorporated genetic material are identified and selected for. Endothelial cells containing incorporated genetic material are referred to as transduced endothelial cells.

In particular, retroviral vectors have been used to
15 stably transduce endothelial cells with genetic material which includes genetic material encoding a polypeptide or protein of interest not normally expressed at biologically significant levels in endothelial cells. The genetic material introduced in this manner also
20 included genetic material encoding a dominant selectable marker. Genetic material including DNA encoding a polypeptide of interest and DNA encoding a dominant selectable marker has been introduced into cultured endothelial cells. Expression of these genes by the
25 endothelial cells into which they have been incorporated (i.e., endothelial cells transduced by the use of retroviral vectors) has also been demonstrated.

A method of transplanting transduced endothelial cells which express the incorporated genetic material
30 they contain is also a subject of the present invention. Transduced endothelial cells of the present invention are

-4-

particularly useful for improving prosthetic implants
(e.g., vessels transplanted from one site in an
individual to another; vessels implanted from another
animal, such as pig, into an individual; vessels made of
05 synthetic materials such as Dacron and polytetra-
fluoroethylene, which are used in vascular reconstructive
surgery) and for providing constitutive delivery of
polypeptides or proteins, useful in prevention and
therapy or treatment, which are presently administered
10 parenterally.

There are many advantages to endothelial cells of
the present invention. For example, they can be designed
to improve the characteristics of endothelial cell-lined
prosthetic implants by enhancing or improving the ability
15 of endothelial cells to seed or bind to the inner surface
of the implant; by modifying the endothelial cells used
to line an implant so that they will grow; or by
overcoming the problem, encountered with presently-
available implants, of smooth muscle cell growth at the
20 implant ends, which results in narrowing and, ultimately,
closing off of the ends. In addition, the transduced
endothelial cells of the present invention can be used to
provide a drug, hormone or enzyme delivery system.

For example, prosthetic arterial grafts are often
25 used to replace diseased arteries which perfuse vital
organs or limbs. However, the currently available grafts
are usually made of synthetic material and are subject to
many complications, the worst of which is a high rate of
re-stenosis or occlusion. Animal studies suggest that
30 lining the graft with autologous endothelial cells prior
to implantation may decrease, but not prevent, graft
reocclusion with its attendant morbid consequences.
Endothelial cells can be modified according to the method

of the present invention in a way that improves their
performance in the context of an implanted graft.
Examples include secretion or expression of a
transmembrane thrombolytic agent to prevent intraluminal
05 clot formation, secretion of an inhibitor of smooth
muscle proliferation to prevent luminal stenosis due to
smooth muscle hypertrophy, and expression and/or
secretion of an endothelial cell mitogen or autocrine
factor to stimulate endothelial cell proliferation and
10 improve the extent of rate of or duration of the
endothelial cell lining of the graft lumen.

An additional application is to cover the surface of
prosthetic heart valves with transduced endothelial cells
in an attempt to improve the performance/longevity of the
15 artificial valves.

A vascular implant lined with endothelial cells
which include incorporated genetic material encoding a
polypeptide of interest synthesizes the encoded
polypeptide and thus serves as a continuous delivery
20 system for that polypeptide. In this way, the hormone or
other polypeptide is secreted directly into the blood-
stream of the individual receiving the implant.

An important advantage is that the genetically
engineered endothelial cells of the present invention can
25 be used to administer therapeutic proteins (e.g.,
hormones, enzymes, clotting factors) which are presently
administered intravenously, intramuscularly or
subcutaneously. In addition, there is no need for
extensive (and often costly) purification of the
30 polypeptide before it is administered to an individual,
as is generally necessary with an isolated polypeptide
(e.g., insulin). Endothelial cells modified according to

-6-

the present invention produce the polypeptide hormone as
it would normally be produced.

Another advantage to the use of genetically
engineered endothelial cells lining prosthetic arterial
05 implants is that one can target the delivery of
therapeutic levels of a secreted product to a specific
organ or limb. The secreted product will be delivered to
tissue perfused by the grafted artery in high
concentrations, thereby achieving a desired effect to a
10 targeted anatomical location. This product will then be
diluted to nontherapeutic levels in the venous
circulation during its return to the heart. For example,
one or more vessels containing transduced endothelial
cells of the present invention which produce an
15 angiogenic factor can be implanted into the leg of an
individual whose circulation is compromised (e.g., as a
complication of diabetes mellitus) resulting in
inadequate blood flow to the foot. This will result in a
high concentration of the encoded product (angiogenic
20 factor) close to the site of the implanted vessel, thus
maximizing the therapeutic availability of the product.
As the blood flows from the extremity, the concentration
of angiogenic factor is reduced to nontherapeutic levels.

Another important advantage of the delivery system
25 of this invention is that because it is a continuous
delivery system, the fact that polypeptide hormones have
very short half lives is not a limitation. For example,
the half life of human growth hormone (HGH) is
approximately 19 minutes, of parathyroid hormone,
30 approximately 2½ to 5 minutes and, of native insulin
(pure insulin), approximately 3 to 4 minutes.

Because genes can be introduced into endothelial
cells using a retroviral vector, they can be "on"
(subject to) the retroviral vector control; in such a
case, the gene of interest is transcribed from a
05 retroviral promoter. A promoter is a specific nucleotide
sequence recognized by RNA polymerase molecules that
start RNA synthesis. Alternatively, retroviral vectors
having additional promoter elements (in addition to the
promoter incorporated in the recombinant retrovirus)
10 which are responsible for the transcription of the
genetic material of interest, can be used. For example,
a construct in which there is an additional promoter
modulated by an external factor or cue can be used,
making it possible to control the level of polypeptide
15 being produced by the endothelial cells by activating
that external factor or cue. For example, heat shock
proteins are proteins encoded by genes in which the
promoter is regulated by temperature. The promoter of
the gene which encodes the metal-containing protein
20 metallothionine is responsive to cadmium (Cd^{++}) ions.
Incorporation of this promoter or another promoter
influenced by external cues also makes it possible to
regulate the production of the polypeptide by the
engineered endothelial cells.

25 <u>Brief Description of the Drawings</u>
Figure 1 is a schematic representation of a wild
type murine leukemia virus (retroviral) genome.
Figure 2 is a schematic representation of retroviral
vectors, each having a recombinant genome, useful in the

-8-

present invention. Figure 2a is pLJ and Figure 2b is
pEm.

Figure 3 is a schematic representation of the
construction of a recombinant retroviral vector, using
05   the pLJ vector represented in Figure 2a and the human
parathyroid hormone gene.

Figure 4 includes pictures of bovine aortic
endothelial cells infected with a retrovirus that
expresses low density lipoprotein receptor (LDLR) and
10   analyzed for the uptake of fluorescent labeled LDL.
Panel A - uninfected bovine aortic endothelial cells
under phase contrast; Panel B - uninfected bovine aortic
endothelial cells under fluorescent illumination; Panel C
- infected bovine aortic endothelial cells under phase
15   contrast; Panel D - infected bovine aortic endothelial
cells under fluorescent illumination.

Figure 5 includes pictures of bovine aortic
endothelial cells infected with a retrovirus that
expresses B-galactosidase and analyzed for its expression
20   using an _in situ_ histochemical stain. Panel A - un-
infected bovine aortic endothelial cells stained for
beta-galactosidase activity; Panel B - infected
unselected bovine aortic endothelial cells stained for
beta-galactosidase activity.

25   Detailed Description of the Invention
Genetic material of interest has been incorporated
into endothelial cells and expressed in the resulting
genetically engineered endothelial cells. Genetic
material of interest incorporated into endothelial cells

according to the method described can be any selected DNA of interest (e.g., all or a portion of a gene encoding a product of interest) or any selected RNA of interest. For example, it can be DNA or RNA which is present and

05 expressed in normal endothelial cells; DNA or RNA which does not normally occur in endothelial cells; DNA or RNA which normally occurs in endothelial cells but is not expressed in them at levels which are biologically significant (levels sufficient to produce the normal

10 physiological effects of the protein or polypeptide it encodes); DNA or RNA which occurs in endothelial cells and has been modified in such a manner that it can be expressed in such cells; and any DNA or RNA which can be modified to be expressed in endothelial cells, alone or

15 in any combination thereof. This genetic material of interest is referred to herein as incorporated genetic material. Endothelial cells of the present invention express the incorporated genetic material. The incorporated genetic material (i.e., DNA or RNA)

20 expressed by endothelial cells of the present invention is genetic material encoding a polypeptide or a protein of interest (genetic material of interest). Endothelial cells of the present invention can also include and express a gene encoding a selectable marker.

25 For example, genetic material of interest which is DNA normally present in and expressed by human endothelial cells can be incorporated into abnormal endothelial cells (e.g., which contain an abnormal quantity of the DNA or which contain but do not express

30 such material adequately), with the result that they are able to produce the desired protein or polypeptide.

-10-

In another example, which has been carried out,
genetic material encoding a hormone has been introduced
into endothelial cells by exposing them to media that
contains a virus having a recombinant genome (i.e., by
05 infecting them). The media used is obtained by
harvesting media in which producer cells (e.g., a Psi am
or amphotropic producer) have been grown. That is,
producer cells have been grown in tissue culture to a
confluent density in Dulbecco's Modified Eagle's medium
10 (DME) with 10% calf serum (CS) and penicillin and
streptomycin. Fresh media is added and subsequently
(e.g., approximately 12 hours later), the media is
harvested. Approximately 10 ml of media is harvested
from a 10 cm plate of confluent producer cells. The
15 spent media (or viral stock) is filtered through a 0.45
micron Millipore filter to remove detached producer cells
and is used immediately to infect cells or is stored at
-70° C. Media is removed from a subconfluent plate of
endothelial cells (recipient endothelial cells) and
20 quickly replaced with viral stock (e.g., 5 ml/10 cm.
plate) containing 8 mcg/ml. of Polybrene (Aldrich).
Subsequently (e.g., approximately 12 hours later), this
is removed and replaced with fresh media. Thus, the
media used is a viral supernatant. The recombinant
25 genome of the infectious virus includes the genetic
material of interest, which is incorporated into
endothelial cells. The recombinant genome can also have
genetic material encoding a dominant selectable marker.
    As a result, endothelial cells were made which
30 express a polypeptide not normally expressed by such

cells at biologically significant levels and, optionally, a dominant selectable marker.

In particular, endothelial cells are exposed to media containing infectious virus produced in Psi am cells; the infectious virus contain a recombinant genome having the genetic material of interest. The recombinant genome in one instance includes genetic material encoding human parathyroid hormone (hPTH). Optionally, it can also include a gene encoding a dominant selectable marker (e.g., the neo gene which encodes neomycin resistance in bacteria and G418 resistance in mammalian cells). As a result, the endothelial cells are transduced -- that is, the genetic material of interest (in this case, DNA encoding hPTH and, optionally, the neo gene) is stably introduced into the endothelial cells. The transduced endothelial cells express the encoded hPTH and, if the neo gene is present, also express it, resulting in cells having the selectable trait.

Alternatively, endothelial cells are transduced as a result of exposure to media containing infectious virus in which the recombinant genome includes genetic material of interest (e.g., any gene of interest or a portion thereof, RNA of interest). As described below, endothelial cells have been transduced with a gene which codes for a secreted product (human parathyroid hormone or hPTH); a gene which codes for a membrane receptor (low density lipoprotein receptor or LDLR); and a gene coding for an intracellular enzyme (beta-galactosidase).

Endothelial cells expressing the incorporated genetic material are grown to confluence in tissue culture vessels; removed from the culture vessel in which

-12-

they were grown; and introduced into or implanted into
the body. They can be introduced into the recipient by
one of several methods. For example, transduced
endothelial cells can be seeded onto the lumen of a
05 prosthetic vessel. The endothelial cells will then grow
to confluence, covering the lumen of the vessel. They
form the lining of what has been called a neointima (a
well organized structure that resembles the intima and
media of a native vessel (i.e., a covering of endothelial
10 cells with deeper layers of smooth muscle cells).

Alternatively, they can be grafted onto a blood
vessel in vivo through the use of a catheter. It is also
possible they can be introduced into body cavities which
are lined by serosal membranes, such as the peritoneal
15 cavity, the pleural space, and the pericardial space. In
this case, the endothelial cells would seed the serosal
lining and secrete the product into the cavity. The
product would then be absorbed via the lymphatic system.

Another example which has been carried out is the
20 implantation into dogs of vascular grafts that have been
seeded with autologous retrovirus-transduced endothelial
cells. Replication-defective retroviruses with
amphotropic host range have been used to stably introduce
a reporter gene into the genome of the endothelial cells.
25 The lac Z gene has been used as the reporter gene because
its product of expression, beta-galactosidase, can be
detected in-situ through the use of enzyme histochemical
assays that stain the cells cytoplasm blue.

External jugular veins harvested from adult mongrel
30 dogs were used as a source of endothelial cells which

were plated _in vitro_ during a 10-14 day period by two
serial passages. Cells from each animal were divided
into two aliquots and were either infected with a
replication defective retrovirus or mock infected. Small
05 diameter dacron grafts were seeded at subconfluent
densities with endothelial cells by the autologous clot
method and surgically implanted as carotid interposition
grafts into the dog from which the cells were harvested;
each dog received a graft seeded with the genetically
10 modified cells and a contralateral graft seeded with MOCK
infected cells. Five weeks after implantation, the
grafts were harvested and analyzed.

Cells were enzymatically harvested from the luminal
surface of a portion of the graft to permit a more
15 detailed characterization. Primary cultures of cells
were established and expanded _in vitro_ for approximately
2-3 weeks prior to analysis. Genetically modified
endothelial cells were identified in this population by
Southern analysis and by the cytochemical assay for
20 viral-expressed beta-galactosidase. The majority of
cells harvested from the graft and expanded _in vitro_
retained differentiated endothelial function (less than
95%). However, the proportion of cells that expressed
viral directed beta-galactosidase or contained proviral
25 sequences was consistently decreased 2-10 fold when
compared to the cultures that were analyzed at the time
of seeding. This disparity is due in part to the partial
repopulation of grafts with endogenous cells by growth
through interstices or from the anastomoses.
30 The results of this study demonstrate the
feasibility of implanting vascular grafts seeded with

-14-

autologous genetically modified endothelial cells. The
transduced cells persist on the lumen of the graft for at
least five weeks and the transferred gene continues to
function.

05 Isolation of Endothelial Cells
        The isolation and maintenance of endothelial cells
from capillaries and large vessels (e.g., arteries,
veins) of many species of vertebrates has been well
described in the literature. For example, McGuire and
10 Orkin describe a simple procedure for culturing and
passaging endothelial cells from large vessels of small
animals. McGuire, R.W. and R.W. Orkin, Biotechniques,
5:546-554 (1987).
        Frequently, calf aorta is the source of endothelial
15 cells, as was the case in the initial work described
herein. A typical protocol for isolation of endothelial
cells from a large artery is described below. Sections
of aortas freshly harvested are placed under sterile
conditions, in a solution containing collagenase (e.g.,
20 0.5 mg/ml) for 15-20 minutes at 37°C. The aortas are
then rinsed twice with tissue culture medium (e.g., RPMI
1640) and the lumenal sheet of endothelial cells is
removed in complete medium (RPMI containing 15 mm Hepes,
pH 7.4, penicillin/streptomycin and 20% fetal calf serum)
25 by gentle agitation, according to the method of Booyse et
al.. Booyse, F.M. et al., Thrombosis Diath. Haemorrh.,
34:825-839 (1975). The cell patches are transferred to
tissue culture flasks in complete medium.
        The cells divide and eventually cover the plate;
30 when the plate is confluent, the cells can be passaged

-15-

using standard tissue culture techniques. The purity of
the cultures is assessed by uptake of fluorescent labeled
acetylated LDL which is specifically taken up by
endothelial cells. If the cultures are not pure (i.e.,
05 contaminated with cells other than endothelial cells,
such as smooth muscle cells or fibroblasts), endothelial
cells are cloned by limiting dilution and expanded to
yield pure cultures. The life span of endothelial cells
is limited in culture but varies markedly, depending on
10 the anatomical source and donor animal. Bovine aortic
endothelial cells have been maintained in culture for at
least 15-20 passages.

Retroviral Vectors
        Retroviruses are RNA viruses; that is, the viral
15 genome is RNA. This genomic RNA is, however, reverse
transcribed into a DNA intermediate which is integrated
very efficiently into the chromosomal DNA of infected
cells. This integrated DNA intermediate is referred to
as a provirus. As shown in Figure 1, the retroviral
20 genome and the proviral DNA have three genes: the gag,
the pol and the env, which are flanked by two long
terminal repeat (LTR) sequences. The gag gene encodes
the internal structural (nucleocapsid) proteins; the pol
gene encodes the RNA-directed DNA polymerase (reverse
25 transcriptase); and the env gene encodes viral envelope
glycoproteins. The 5' and 3' LTRs serve to promote
transcription and polyadenylation of virion RNAs.
        Adjacent to the 5' LTR are sequences necessary for
reverse transcription of the genome (the tRNA primer
30 binding site) and for efficient encapsidation of viral

-16-

RNA into particles (the Psi site). Mulligan, R.C., _In:
Experimental Manipulation of Gene Expression_, M. Inouye
(ed), 155-173 (1983); Mann, R., _et al._, _Cell_, 33:153-159
(1983); Cone, R.D. and R.C. Mulligan, _Proceedings of the
05 National Academy of Sciences, U.S.A._, 81:6349-6353
(1984).

     If the sequences necessary for encapsidation (or
packaging of retroviral RNA into infectious virions) are
missing from the viral genome, the result is a _cis_ defect
10 which prevents encapsidation of genomic RNA. However,
the resulting mutant is still capable of directing the
synthesis of all virion proteins. Mulligan and
co-workers have described retroviral genomes from which
these Psi sequences have been deleted, as well as cell
15 lines containing the mutant stably integrated into the
chromosome. Mulligan, R.C., _In: Experimental Manipu-
lation of Gene Expression_, M. Inouye (ed), 155-173
(1983); Mann, R., _et al._, _Cell_, 33:153-159 (1983); Cone,
R.D. and R.C. Mulligan, _Proceedings of the National
20 Academy of Sciences, U.S.A._, 81:6349-6353 (1984). The
teachings of these publications are incorporated herein
by reference.

     As described by Mulligan and co-workers, the Psi 2
cell line was constructed in the following manner: A
25 mutant murine leukemia virus (MuLV) genome in which the
region or the genome implicated in the encapsidation of
viral RNA into virions is deleted (the Psi sequence in
Figure 1) was constructed. This genome was stably
introduced in NIH3T3 cells by DNA cotransformation and
30 stable transformants that produced all of the viral
proteins used for encapsidation, yet budded noninfectious

particles, were isolated. The mutant of MuLV was
constructed by deleting 351 nucleotides from an
infectious proviral DNA clone between the putative env
mRN4 5' splice site and the AUG that initiates the coding
05 sequence for Pr 65^{gag}. The deletion was made from a Bal
1 site to a Pst 1 site and a Hind III site was generated
at the point of deletion.

pMOV. (pMOVPsi^{-}) was constructed as follows: Three
purified DNA fragments were ligated together to construct
10 pMOV Psi^{-}. The first was obtained by digesting pMOV Psi^{+}
with Xho I to completion, followed by partial digestion
with Eco RI. Chumakov, I. et al., Journal of Virology,
42:1088-1098 (1982). The fragment extending from the Xho
I site at 2.0 U in MuLV, through the 3' LTR, 3' mouse
15 flanking sequence, all of pBR322, and ending at the Eco
RI site was purified from an agarose gel after
electrophoretic separation. Vogelstein, B. and D.
Gillespie, Proceedings of the National Academy of
Sciences, USA, 761:615-619 (1979). The second fragment
20 was obtained by digestion of pMOV Psi^{+} with Bal I to
completion followed by purification of the fragment
extending from the Bal I site in pBR322 through 5' mouse
flanking sequence and 5' LTR to the Bal I site located at
0.7 U of MuLV. Hind III linkers (Collaborative Research)
25 were then blunt-ligated to this fragment with T4 DNA
ligase, and the fragment was digested with excess Hind
III and Eco RI. The LTR-containing fragment was purified
from an agarose gel after electrphoretic separation. The
third fragment present in the final ligation reaction was
30 obtained from pSV2-gag/pol where the gag/pol region of
MuLV had been subcloned into pSV2. Mulligan, R.C. and P.

-18-

Berg, Science, 209:1422-1427 (1980). pSV2-gag/pol was
digested to completion with Xho I and Hind III and the
fragment extending from the Hind III site (changed from
the Pst I site at 1.0 U of MuLV) to the Xho I site at 2.0
05 of MuLV was purified from an agarose gel following
electrophoretic separation. These three DNA fragments
were then mixed in equimolar amounts at a total DNA
concentration of 50 ug/ml. in ligase buffer (50 mM
Tris-HCl [pH 7.8], 10 mM MgCl2, 20 mM dithiothreitol, 1.0
10 mM ATP, 50 ug/ml. bovine serum albumin) and incubated
with T4 DNA ligase for 18 hr. at 15°C. E. coli HB101 was
transfected with the ligated DNA, and ampicillin
resistant transfectants were obtained. The plasmid DNA
obtained from a number of transformants was screened for
15 the desired structure by digestion, with appropriate
restriction endonucleases and electrophoresis through
agarose gels. Davis, R.W. et al., Methods in Enzymology,
65:404-411 (1980).
        Cell lines containing the Psi⁻ mutant stably
20 integrated into the chromosome were made by
cotransfection of pMOV-Psi⁻ and pSV2gpt, a SV40 hybrid
vector capable of XG PRT expression. Mulligan, R.C. and
P. Berg, Science, 209:1422-1427 (1980). Cells from gpt⁺
colonies obtained in this way were cloned and established
25 into three lines: Psi-1, Psi-2, and Psi-3.
        The Psi 2 cell line described by Mulligan and
co-workers was created by transfecting NIH 3T3
endothelial cells with pMOV-Psi⁻, which is an ecotropic
Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-Psi⁻
30 expresses all the viral gene products but lacks the Psi
sequence, which is necessary for encapsidation of the

viral genome. pMOV-Psi⁻ expresses an ecotropic viral
envelope glycoprotein which recognizes a receptor present
only on mouse (and closely related rodent) cells.

Another cell line is the Psi am line, which are
05 Psi-2-like packaging cell lines. These Psi-am cell lines
contain a modified pMOV-Psi-genome, in which the eco-
tropic envelope glycoprotein has been replaced with   .
envelope sequences derived from the amphotropic virus
4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology,
10 19:19-25 (1976). As a result, they are useful for
production of recombinant virus with amphotropic host
range. The retrovirus used to make the Psi am cell line
has a very broad mammalian host range (an amphotropic
host range) and can be used to infect human cells. If
15 the recombinant genome has the Psi packaging sequence,
the Psi-am cell line is capable of packaging recombinant
retroviral genomes into infectious retroviral particles.
Cone, R. and Mulligan, R., Proceedings of the National
Academy of Sciences, USA, 81:6349-6353 (1984).

20   The retroviral genome has been modified by Cone and
Mulligan for use as a vector capable of introducing new
genes into cells. As shown in Figure 2, the gag, the pol
and the env genes have all been removed and a DNA segment
encoding the neo gene has been inserted in their place.
25 The neo gene serves as a dominant selectable marker. The
retroviral sequence which remains part of the recombinant
genome includes the LTRs, the tRNA binding site and the
Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062
(1984).

30   Additional vector constructions which have been used
in producing transduced endothelial cells of the present

-20-

invention are represented in Figure 2 and are described
in detail below.

pLJ. The characteristics of this vector have been
described in Korman, A.J. et al., Proceedings of the
05 National Academy of Sciences, USA 84:2150 (1987). This
vector is capable of expressing two genes: the gene of
interest and a dominant selectable marker, such as the
Neo gene. The gene of interest is cloned in direct
orientation into a BamHI/SmaI/SalI cloning site just
10 distal to the 5' LTR, while, the Neo gene is placed
distal to an internal promoter (from SV40) which is
farther 3' than is the cloning site (is located 3' of
the cloning site). Transcription from PLJ is initiated
at two sites: 1) the 5' LTR, which is responsible for
15 expression of the gene of interest and 2) the internal
SV40 promoter, which is responsible for expression of the
neo gene. The structure of pLJ is represented in Figure
2a.

Vector pLJ is represented in Figure 2a. In pLJ, the
20 genetic material of interest is inserted just following
the 5' LTR. Expression of this genetic material is
transcribed from the LTR and expression of the neo gene
is transcribed from an internal SV40 promoter.

pEm. In this simple vector, the entire coding
25 sequence for gag, pol and env of the wild type virus is
replaced with the gene of interest, which is the only
gene expressed. The components of the pEm vector are
described below. The 5' flanking sequence, 5' LTR and
400 bp of contiguous sequence (up to the BamHI site) is
30 from pZIP. The 3' flanking sequence and LTR are also
from pZIP; however, the ClaI site 150 bp upstream from

the 3' LTR has been ligated with synthetic <u>BamHI</u> linkers
and forms the other half of the BamHI cloning site
present in the vector. The <u>HindIII</u>/<u>EcoR1</u> fragment of
pBR322 forms the plasmid backbone. This vector is
05 derived from sequences cloned from a strain of Moloney
Murine Leukemia virus. An analogous vector has been
constructed from sequences derived from the
myeloproliferative sarcoma virus. The structure of pEM
is represented in Figure 2b.
10   Vectors without a selectable marker can also be used
to transduce endothelial cells with genetic material of
interest. Such vectors are basically simplifications of
the vectors previously described, in which there is such
a marker. Vector pEm is represented in Figure 2b; as
15 represented, the main components of the vector are the 5'
and 3' LTR, and the genetic material of interest,
inserted between the two LTRs.

<u>Introduction of Genetic Material into Endothelial cells</u>
<u>and Assessment of Expression of the Genetic Material</u>
20   A cell line which produces recombinant amphotropic
retrovirus having a recombinant genome is used to infect
endothelial cells. As described above, the recombinant
genome can include a variety of components, but in
general is comprised of two LTRs and, in place of the
25 <u>gag</u>, the <u>pol</u> and the <u>env</u> sequences, a second promoter
sequence. In some cases, it also includes a gene
encoding a selectable marker (e.g., <u>neo</u>).
      Viral stocks, to be used in introducing genetic
material of interest into endothelial cells, are
30 harvested, as described above, supplemented with 8

-22-

micrograms per ml. (mcg/ml.) of Polybrene (Aldrich) and
added to the culture of endothelial cells. If the titer
of the virus is high (e.g., approximately 10^6 cfu
per ml.), then virtually all endothelial cells will be
05 infected and no selection (e.g., of endothelial cells
into which the vector, including the recombinant genome,
has been introduced) is required. If the titer is very
low, then it is necessary to use a retroviral vector that
has a selectable marker, such as neo or his. If a
10 selectable marker is used, after exposure to the virus,
the cells are grown to confluence and split into
selective media (e.g., media containing G418 if the
selectable marker is neo, media containing histidinol and
no histidine if the selectable marker is his).

15   The neo gene is a bacterial gene derived from the
transposon Tn5, which encodes neomycin resistance in
bacteria and resistance to the antibiotic G418 in mamma-
lian cells. This neo gene acts as a dominant selectable
marker; its presence in a mammalian cell converts the
20 cell into one which will grow in the presence of G418.
(If it is not present, the cell dies in the presence of
G418.) As a result, the presence of this gene in a
mammalian cell can be determined by culturing cells in
media which contains G418. The recombinant retrovirus
25 having this recombinant genome is referred to as the neo
virus.

The recombinant retroviral vectors having the neo
gene also have a cloning site. As a result, genetic
material of interest can be introduced into the vector,
30 incorporated into endothelial cells and expressed by
endothelial cells transduced with the recombinant

retrovirus (referred to as endothelial cells having
incorporated genetic material).

It should be possible to express virtually any gene
of interest in endothelial cells by means of a retroviral
05 vector. Retroviral vectors that express genes that
encode three different classes of proteins have been
constructed: a secreted hormone polypeptide (human PTH,
or hPTH), a membrane receptor (receptor for LDL, or
LDLR), and an intracellular enzyme (beta-galactosidase).
10 Efficient expression of the recombinant viruses when
incorporated into endothelial cells has been demonstrated
and is described in detail in the examples.

-24-

Introduction of Genetic Material Encoding Other Proteins
or Polypeptides

      Genes encoding other proteins or polypeptides can
also be introduced into endothelial cells by means of an
05 appropriate retroviral vector. For example, a gene
encoding human growth hormone (HGH) or a gene encoding
insulin can be introduced into endothelial cells. Such
genes can be introduced into endothelial cells, alone or
in combination with a gene encoding a selectable marker,
10 such as the neo gene. HGH is a polypeptide of about
29,000 Daltons which is normally secreted only by the
hypothalamus. Insulin is a polypeptide which regulates
glucose levels in the bloodstream.

      These genes, as well as others can be introduced
15 into endothelial cells in the same manner as described
above for the hPTH gene and the resulting transduced
endothelial cells can be implanted into or applied to an
appropriate site on the body.

Use of Other Dominant Selectable Markers in the Intro-
20 duction of Genetic Material Encoding Polypeptides

      It is also possible to use dominant selectable
markers other than the neo gene to introduce genetic
material into endothelial cells. For example, the His D
gene can be used for this purpose. The His D gene is a
25 bacterial gene from Salmonella and encodes histidinol
dehydrogenase, a polypeptide which converts histidinol to
histidine. Histidine is an essential amino acid; histi-
dinol is an alcohol analogue of histidine and can be con-
verted to histidine under the proper metabolic condi-
tions. If cells are grown in media containing histidinol

but lacking histidine, those cells having the His D gene
can convert histidinol to histidine. Because histidine
is essential to their function, those cells which have
the His D gene (and thus can make histidine) will survive
05 and those lacking the gene will not.

A retrovirus vector having the His D gene has been
used to infect keratinocytes. The keratinocytes contain-
ing His D gene were selected by growing these cells in
media lacking histidine but containing histidinol. As
10 expected, keratinocytes having the His D gene formed
colonies and grew to confluence; those lacking the gene
did not. In fact, such cells occurred at a much higher
frequency than those in which the neo gene was included.
These same techniques are useful in selecting endothelial
15 cells containing DNA of interest.

As a result of this work, it is also possible to use
independent dominant selectable markers (e.g., the neo
gene and the His D gene) to incorporate new genetic
material into endothelial cells. In the case of
20 polypeptides which have two different subunits, for
example, separate dominant selectable markers can be used
to introduce the genetic information encoding the two
subunits. In addition, two or more dominant selectable
markers can be used in the case of polypeptides which
25 need to be specifically cleaved or processed in order to
become active (e.g., insulin and parathyroid hormone). A
gene encoding the necessary processing enzyme can be
introduced along with the gene encoding the polypeptide
hormone requiring such processing. This would enable
30 endothelial cells to process that polypeptide hormone.

-26-

Other Vehicles for the Introduction of Genetic Material
of Interest into Endothelial cells

It is also possible to use vehicles other than
retroviruses to genetically engineer or modify
05 endothelial cells. Genetic information of interest can
be introduced into endothelial cells by means of any
virus which can express the genetic material of interest
in such cells. For example, SV40, herpes virus,
adenovirus and human papilloma virus can be used for this
10 purpose.

It is also possible to introduce genetic material of
interest into endothelial cells in such a manner that it
is not incorporated stably into the recipient cells, but
is expressed episomally (remains distinct or separate
15 from the recipient cell genome).

Uses of Endothelial Cells Having Incorporated Genetic
Material
        Improvement of Performance of Vascular Grafts or
        Implants
20   Many important disease states involve stenosis or
occlusion of arteries that supply vital organs. The
pathologic mechanism most commonly implicated in such
disease states is atherosclerosis. Examples include
angina pectoris and myocardial infarction due to coronary
25 artery disease; transient ischemic attacks and strokes
due to cerebral vascular disease; renal vascular
hypertension, and ultimately, renal failure due to renal
artery stenosis; and claudication of the lower
extremities, which is caused by vascular disease of
30 peripheral arteries and, in its most severe form, can

result in amputation. Unless the agents that predispose
an individual to atherosclerotic lesions are eliminated
(e.g., hypertension, cigarette smoking, and
hypertension), the natural history of these disease
05 states is usually progression of the atherosclerotic
lesions, resulting in permanent damage or death.

An accepted and widely used therapeutic approach to
advanced atherosclerotic disease is to bypass the site of
major stenosis or occlusion with a prosthetic vessel made
10 of synthetic material, such as Dacron. More than 350,000
vascular grafts are implanted each year. One major
problem with this approach is that the prosthetic vessel
is extremely thrombogenic (i.e., it has the propensity to
develop clots), which leads to a very high rate of
15 re-stenosis. It has been possible to reduce this problem
by seeding the lumen of the prosthetic vessels with
autologous endothelial cells; grafts lined with
endothelial cells are presumably less thrombogenic. It
is in this setting that modified endothelial cells would
20 be particularly useful.

Endothelial cells can be genetically modified
according to the method of the present invention to
improve their performance in the context of an
endothelial cell-lined prosthetic implant. Existing
25 protocols for using endothelial cell-lined prosthetic
implants are complicated by several significant
technological problems, most of which can be overcome
through the use of genetically engineered endothelial
cells.

30   First, it is impossible to achieve a confluent layer
of endothelial cells lining the graft when cells are

-28-

seeded at subconfluent density. This is due, in part, to
the limited proliferative capacity of endothelial cells
in this milieu. Furthermore, limited quantities of
available autologous endothelial cells preclude the
05 seeding of grafts at confluent densities (using currently
available technology) in most relevant clinical
situations. However, endothelial cells can be
genetically modified to enhance their proliferative
capacity by infecting them with a retrovirus which
10 expresses an endothelial cell mitogen, such as
endothelial cell growth factor. It may be possible to
initially seed an implant with transduced endothelial
cells at subconfluent densities and subsequently achieve
a confluent lining in vitro or in vivo. Many
15 applications of endothelial cell-lined prosthetic
implantation which have been impossible due to limited
quantities of autologous cells may now be feasible. In
addition, the modified endothelial cells may exhibit a
selective advantage over competing cells (such as smooth
20 muscle cells or endogenous endothelial cells), thereby
preventing overgrowth of the transduced endothelial cells
by the competing cells.
       An additional problem with endothelialized implants
is that the lumen of the prosthetic vessel undergoes
25 progressive narrowing due to the proliferation of smooth
muscle cells between the wall of the prosthesis and the
luminal surface. One way in which this can be prevented
is to introduce into the endothelial cells a gene that
secretes a product which inhibits the growth of smooth
30 muscle cells. Many types of autocrine:paracrine growth
factors that control proliferation of mesenchymal and/or

epithelial cells have recently been identified and their genes cloned. Such genes can be introduced into endothelial cells using the method of the present invention. The resulting transduced endothelial cells
05 produce the cell growth inhibitor.

A further technical problem of endothelialized implant protocols is that the binding (plating) efficiency of the endothelial cells to the prosthetic graft is relatively low. Previous attempts to improve
10 this have been directed at modifying the composition of the graft surface and have been of limited success. Using the method of the present invention, it is possible to introduce into endothelial cells a gene which codes for a membrane receptor. The lumen of the prosthetic
15 vessel can then be coated with the ligand for the receptor, thereby facilitating binding of endothelial cells to the luminal surface through the membrane receptor/ligand interaction.

Genetically engineered endothelial cells of the
20 present invention can be used to decrease the thrombogenicity of endothelial cell-lined prosthetic grafts. The mechanism of clot formation is believed to be platelet adhesion followed by deposition of fibrin and propagation of clot. This could be minimized or possibly
25 eliminated by seeding the grafts with genetically engineered endothelial cells that secrete a thrombolytic agent (e.g., an agent which dissolves clots, such as tissue plasminogen activator (TPA) or streptokinase). An even more effective approach would be to design a
30 modified form of TPA which attaches to the endothelial cell membrane, rather than being secreted. In this case,

-30-

the thrombolytic agents would be concentrated in the
vessel lumen, where they would have the greatest effect.

Use of Modified Endothelial Cells to Deliver a Product to
a Limb or Organ

05   This invention can also be used to introduce into
endothelial cells genes that secrete factors which would
be beneficial to the limb or organ perfused by the artery
containing the prosthesis. For example, a common
clinical problem is the presence of extensive narrowing

10 of small vessels distal to the site of the prosthetic
vessel. This is characteristic of the vascular disease
associated with diabetes mellitus. Revascularization of
the larger vessels with implants leads to incomplete
reconstitution of perfusion to the affected limb or

15 organ. One could effect increased vascular flow to the
compromised tissue by promoting the development of
collateral circulation from contiguous tissue which is
not compromised. This occurs naturally to a limited
extent. However, the whole process of collateralization

20 could be accelerated and more fully developed if
endothelial cells in the affected area secrete a factor
or factors that promote angiogenesis. The genes for many
of these angiogenic factors have been cloned and can be
introduced into endothelial cells (e.g., using a

25 retrovirus in which the genome includes the gene of
interest). The delivery of the angiogenic factor(s)
would be targeted to the organ or limb supplied by the
artery containing the graft. The effective concentration
of the factor would be relatively high in the affected

30 tissue, but low in general circulation because it would

be diluted in the venous circulation upon its return to
the heart. Thus, it should be possible to obtain a
therapeutic effect in the diseased organ or limb without
systemic side effects.

05   Another way of further promoting vascular flow to a
compromised organ or limb is to maximally dilate all
afferent vessels. Attempts to do this with oral or
parenteral medicines have resulted in little therapeutic
benefit, accompanied by many systemic side effects. This
10 is caused, in part, by the fact that the vasodilator is
not targeted to the appropriate tissue. Endothelial
cells engineered to secrete a potent vasodilator such as
atrial naturetic factor is an alternative approach. In
this application, the affected organ or limb can be
15 perfused with arterial blood which contains a very high
concentration of a vasodilator. This results in an
increase in the overall vascular perfusion. However, the
vasodilator is diluted to nonpharmacologic levels upon
return to the heart, thereby obviating the many systemic
20 side effects of vasodilators that occur when administered
in a nonselective manner.

Use of Modified Endothelial Cells as a Delivery System

The present invention makes it possible to geneti-
cally engineer endothelial cells in such a manner that
25 they produce a selected protein or polypeptide, such as
selected polypeptides and proteins not normally produced
in endothelial cells in biologically significant amounts,
and secrete them into the bloodstream or other area of
the body (e.g., the central nervous system). The
30 endothelial cells formed in this way can serve as a

-32-

continuous drug delivery system to replace present
regimens, which require periodic administration (by
ingestion, injection, etc.) of the needed substance.

For example, it can be used to provide continuous
05 delivery of insulin, which, at the present time, must be
isolated from the pancreas, extensively purified and then
injected into the body by those whose insulin production
or utilization is impaired. In this way, insulin can be
introduced into the body via a continuous drug delivery
10 system and, as a result, there would be no need for daily
injections of insulin.

Genetically engineered endothelial cells can also be
used for the production of clotting factors.
Hemophiliacs lack a protein called Factor VIII, which is
15 involved in clotting. Factor VIII is now administered by
injection. Endothelial cells having genes encoding
Factor VIII can be used to make an implant in which they
produce Factor VIII; as an implant, the tissue would
secrete the factor into the bloodstream.

20   Incorporation of genetic material of interest into
endothelial cells can be particularly valuable in the
treatment of inherited disease and the treatment of
acquired disease. In the case of inherited diseases,
this approach is used to provide genetically modified
25 endothelial cells and other cells which can be used as a
metabolic sink. That is, such endothelial cells would
serve to degrade a potentially toxic substance. For
example, transduced endothelial cells used in treating
urea cycle disorders. Endothelial cells of the present
30 invention can also be used in the treatment of genetic
diseases in which a product (e.g., an enzyme or hormone)

normally produced by the body is not produced or is made
in insufficient quantities. Here, endothelial cells
transduced with a gene encoding the missing or
inadequately produced substance can be used to produce it
05 in sufficient quantities. For example, this can be used
in producing alpha-1 anitrypsin. It can also be used in
the production of Factor VIII and Factor IX, and thus
would be useful in treating hemophilia.

There are many acquired diseases for which treatment
10 can be provided through use of genetically engineered
endothelial cells (i.e., endothelial cells transduced
with genetic material of interest). For example, such
cells can be used in treating anemia, which is commonly
present in chronic disease and often associated with
15 chronic renal failure (e.g., in hemodialysis patients)..
In this case, endothelial cells having incorporated in
them a gene encoding erythropoietin would correct the
anemia by stimulating the bone marrow to increase
erythropoiesis (i.e. production of red blood cells).
20 Endothelial cells of the present invention can also
be used to administer a low dose of tissue plasminogen
activator as an activator to prevent the formation of
thrombi. In this case, endothelial cells having
incorporated genetic material which encodes TPA would be
25 transplanted into an individual in whom thrombus
prevention is desired. This would be useful, for
example, as a prophylactic against common disorders such
as coronary artery disease, cerebrovascular disease,
peripheral vascular occlusive disease, vein (e.g.,
30 superficial) thrombosis, such as seen in pulmonary
emboli, or deep vein thrombosis. Endothelial cells which

-34-

contain DNA encoding calcitonin can be used in the treatment of Paget's Disease, a progressive, chronic disorder of bone metabolism. Present treatment relies on subcutaneous administration of calcitonin.

05   Endothelial cells engineered to produce and secrete interleukins (e.g., IL-1, IL-2, IL-3) can be used in several contexts. For example, the result of some of the therapies now used (e.g., chemotherapy) is induction of neutropenia (the presence of abnormally low numbers of

10 neutrophils in the blood), often caused by direct suppression of the bone marrow. For example, use of virtually all the chemotherapeutic agents, as well as AZT, used in the treatment of (AIDS) Acquired Immune Deficiency Syndrome, results in neutropenia. This

15 condition results in numerous life-threatening infections. In these cases, administration of, for example, IL-3 through implantation of endothelial cells which contain genetic material encoding IL-3 can be used to increase the neutrophil count. In addition, the

20 administration of thrombopoietin, which stimulates the production of platelets, can be used in the treatment of numerous conditions in which platelet count is low. In this case, endothelial cells transduced with the gene for thrombopoietin can be applied to/implanted in an

25 individual; production and secretion of the encoded product will result in stimulation of platelet production.

      Another related application of endothelial cells having incorporated genetic material is in the treatment

30 of AIDS. Interleukin 2 and Interleukin 3, which stimulate the immune system, are potentially valuable in

the treatment of AIDS. They could be delivered by a
vessel implant having endothelial cells which have been
genetically engineered to produce these two polypeptides
(which are now administered by periodic injection).

05   Another use of the present invention is in the
treatment of enzyme defect diseases. In this case the
product encoded by the gene introduced into endothelial
cells is not secreted (as are hormones); rather, it is an
enzyme which remains inside the cell. There are numerous

10 cases of genetic diseases in which an individual lacks a
particular enzyme and is not able to metabolize various
amino acids or other metabolites. The correct genes for
these enzymes could be introduced into an endothelial
cell implant; the implant would then carry out that

15 metabolic function. For example, there is a genetic
disease in which those affected lack the enzyme adenosine
deaminase. This enzyme is involved in the degradation of
purines to uric acid. It might be possible, using the
present invention, to produce an implant capable of

20 producing the missing enzyme at sufficiently high levels
to detoxify the blood as it passes through the area at
which the implant is applied.

The present invention also has veterinary applica-
tions. It can be used, for example, in delivering

25 substances such as drugs (e.g., antibiotics) and hormones
to animals, which would otherwise be provided by being
incorporated into their feed, added to their water or
injected periodically (e.g., daily or less frequently).
Use of the modified endothelial cells of the present

30 invention has the advantage that the tissue formed of the
modified cells can be applied to the animal and will

-36-

provide quantities of the encoded protein on an ongoing
basis, thus eliminating the need for daily/periodic
administration of the substance.

The present invention will now be illustrated by the
05 following examples, which are not intended to be limiting
in any way.

EXAMPLE 1   Production of Human Parathyroid Hormone
in Transduced Endothelial Cells

At the BamHI cloning site of pLJ, genetic material
10 of interest can be inserted. The genetic material of
interest can be DNA as described above.

In particular, a copy of the gene encoding human
parathyroid hormone (hPTH) has been cloned into this
site, (e.g., into pLJ) in the following way: The pLJ
15 plasmid was digested with BamHI and subsequently treated
with the enzyme calf intestinal phosphatase. Following
this, the linear vector was fractionated on argarose gel
and purified, using glass beads. In addition, the BamHI
fragment containing the human PTH gene was prepared from
20 the plasmid described by Hendy et al., which contains a
complete cDNA of human PTH cloned into pBR322. Hendy,
G.N. et al., Proceedings of the National Academy of
Sciences, USA, 78:7365-7369 (1981).

A sub fragment of the PTH cDNA, containing 17 bp of
25 5' untranslated, all coding and 31 bp of 3' untranslated
sequence, was isolated by digesting the initial plasmid
with DdeI and HinfI and isolating the 600bp fragment.
The fragment was incubated with DNA polymerase in the
presence of deoxynucleoside in phosphates, to fill in the

recessed ends. BamHI linkers were ligated to the blunt
ends with T_4 DNA ligase. An authentic BamHI restriction
fragment was generated by digesting this ligation mixture
with BamHI. This was then subcloned into the BamHI site
05 of pBR322, which is the plasmid used as the source of
hPTH in vector construction.

Equal quantities of the pLJ linear backbone and the
BamHI PTH fragment were added together, in the presence
of T4 DNA ligase. The resulting mixture was maintained
10 under conditions appropriate for ligation of the two
fragments. The ligation mixture was used to transform
bacteria HB101, which were then plated onto agar
containing kanamycin. Maniatis, T. et al., Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor
,15 Laboratory, p.p. 250-251, 504; Bolivar, F. and K.
Backman, In: Methods in Enzymology, R. Wu (ed.), Vol. 68,
Academic Press, N.Y. (1979). The resulting colonies were
analyzed for the recombinant plasmid.

Parathyroid hormone is a polypeptide which has a
20 role in the regulation of calcium in the body. Although
the hPTH gene is present in human endothelial cells, it
is not expressed in those cells at biologically
significant levels. Endothelial cells capable of making
a polypeptide hormone such as hPTH, or another substance
25 not normally made by such cells at biologically
significant levels, can be engrafted onto or implanted
into an individual and serve as a continuous supply
system for the hormone, or other substance.

The Psi am cells producing the recombinant virus
30 construct which contained the hPTH-encoding DNA and DNA
encoding a selectable marker (such as the neo gene), were

-38-

used to produce a viral stock, as described above. The
viral stock was harvested; endothelial cells to be
transduced with the virus containing the hPTH gene were
incubated with the stock. In this case, a selectable
05 marker is used to identify and select for transduced
endothelial cells by culturing on media containing G418.
If the viral titer is sufficiently high, essentially all
endothelial cells are infected and selection, using a
selectable marker and appropriate media, is not needed.
10   The ability of endothelial cells transduced with the
recombinant retrovirus having the hPTH gene to express
the hPTH gene has been assessed _in_ _vitro_ as follows:
The _in_ _vitro_ production of hPTH by endothelial cells
established from a bovine aorta and infected with a virus
15 containing the hPTH gene was assessed. The bovine aortic
endothelial cells were derived from an explant from the
aorta of a calf. The cells have a limited life span and
are referred to as secondary cultures.
       Bovine aortic endothelial cells were infected with
20 virus and not selected in neomycin, as described above.
Transduced bovine aortic endothelial cells were seeded
onto 10 cm tissue culture dishes and grown to confluence.
.Fresh culture media (DME with 10% CS and penicillin and
streptomycin) was then added; this point is referred to
25 subsequently as time zero. At the end of 24 hours, the
media was removed and the cells were assayed for the
production of human PTH.
       The aliquots were analyzed for the presence of hPTH
using a radioimmunoassay (Nichols) which measures intact
30 hPTH. The technique is described in Allegro^{TM} Intact
PTH/Immunoassay System for the Quantitative Determination

of Human Intact Parathyroid Hormone in Serum, Nichols
Institute Diagnostics, San Juan Capistrano, CA (36B-2170,
Effective 7/86 Revised), the teachings of which are
incorporated herein by reference. The assay has a

05 sensitivity of approximately one nanogram/milliliter
serum (ng/ml) and was shown to be specific for human PTH
in that it does not cross react with calf PTH. The
results of the experiments are reported as the production
of hPTH, as measured by RIA, over time. Results are

10 shown in Table I.

TABLE I

Production of hPTH in Transduced BAE Cells

Cell	PTH Production
Control BAE[*,]	10 pg/10^6/24h
Transduced BAE	6.3ng/10^6/24h

[*]BAE = bovine aortic endothelial

Endothelial cells from a bovine aorta transduced
with DNA encoding hPTH according to the method of the
present invention have been deposited at the American

20 Type Culture Collection (Rockville, MD) under deposit
number CRL9601, under conditions appropriate to meet the
requirements of the Budapest Treaty.

-40-

EXAMPLE 2   Production of Human LDL Receptor in
                   Transduced Endothelial Cells

        The cDNA for human LDL receptor (LDLR) was inserted
        into the pEM vector. The cDNA for LDLR was prepared for
05   insertion into the pEM as follows. LDLR cDNA was excised
        from the vector pTZ1 (obtained from Dr. Goldstein,
        University of Texas Health Science Center) by digestion
        with HindIII. The 2.6 kb HindIII fragment containing all
        of the LDLR coding sequence was blunt ended with Klenow
10   fragment and BclI oligonucleotide linkers (from NEB) were
        ligated to the cDNA with T4 DNA ligase. Finally, this
        ligation mixture was digested with an excess of the
        enzyme BclI and the fragment was fractionated on an
        agarose gel and purified. This was inserted into the
15   BamHI cloning site of pEM as follows. pEM was digested
        with BamHI and the linearized plasmid was digested with
        calf intestinal phosphatase. Equal quantities of the
        linkered LDLR insert and pEM backbone were mixed and
        ligated with T4 DNA ligase. The ligation mixture was
20   used to transform HB101 and ampicillin resistant colonies
        were analyzed for the appropriate retroviral vector. The
        pEM-LDLR vector was transfected into the Psi-am cell line
        and clones that produced high quantity of recombinant
        virus were identified. Viral stocks were used to infect
25   cultures of bovine aortic endothelial cells as described
        for PTH.

        In vitro assessment of LDLR expression was carried
        out as follows: confluent plates of control or
        transduced bovine aortic endothelial cells were incubated
30   with LDL that had been conjugated with a fluorescent

label (obtained from Biomedical Tech Inc., Stoughton, MA)
at a concentration of 10 ug/ml for approximately 8 hours.
Following this, the cells were washed with PBS and fixed
in 0.5% gluteraldehyde in PBS. They were then visualized
05 under a fluorescent microscope for the uptake of
fluorescent labeled LDL (*LDL). The results of this
experiment are presented in Figure 4. Briefly, the level
of endogenous LDLR is low, as evidenced by the relative
lack of *LDL uptake in uninfected cultures (See 4B).
10 However, in cultures infected with the LDLR virus,
approximately 30% of all cells take up detectable
quantities of *LDL, thereby documenting efficient
expression of the exogenous LDLR (See 4D).

EXAMPLE 3   Production of Beta-galactosidase in
15   Transduced Endothelial Cells

The gene endocing beta-galactosidase from E. coli
was inserted into pLJ and this vector was transfected
into the Psi-am cell line, resulting in production of
high titer retroviral stocks. The construction of this
20 vector and isolation of the producer cell line has been
described by Price and co-workers. Price, J. et al.,
Proceedings of the National Academy of Sciences, USA,
84:156-160 (1987). Stocks of virus encoding the
beta-galatosidase gene were used to infect bovine aortic
25 endothelial cells, as described earlier. The infected
cultures were analyzed for beta-galatosidase expression
using an in situ histochemical stain. See Price et al.
above. Cultures were analyzed before and after selection
in G418. The retroviral vector used in this experiment

expresses both beta-galactosidase and the <u>neo</u> gene which
confers resistance to G418. The histochemical stain was
performed as described by Price et al. Briefly, cell
cultures were fixed in 0.5% gluteraldehyde in PBS for 5

05 minutes, washed with PBS and exposed to the reaction
mixture for at least 12 hours. The reaction mixture
contains a substrate for beta-galatosidase which, when
hydrolyzed, turns blue and precipitates in the cell. As
a result, any cell expressing the viral encoded

10 beta-galactosidase will turn blue. The results of this
experiment are presented in Figure 5. No beta-galacto-
sidase activity is detected in cultures that were not
exposed to virus (Figure 5A); infected cultures
demonstrate beta-galactosidase activity in about 30% of

15 the cells (Figure 5B). These transduced cells are
selected for by incubating them in the presence of G418.

EXAMPLE 4   Production of Beta-galactosidase in
                     Transduced Endothelial Cells on the
                     Surface of Vascular Grafts Implanted
20   Into Dogs

Endothelial cells were enzymatically harvested from
external jugular veins of adult mongrel dogs which
weighed 20-25 kg. Cells from each animal were divided
into 2 aliquots; one to be infected with a replication

25 defective retrovirus (see below) and the other to be mock
infected. The enzymatically harvested cells were used to
establish primary cultures. Endothelial cells were
plated on to fibronectin coated flasks and maintained in
M199 Medium supplemented with 5% plasma derived equine

serum, penicillin, streptomycin, heparin and ECGF during
two serial passages over a 10-14 day period.

During this time period, cells were exposed to fresh
stocks of virus supplemented with polybrene (8 ug/ml)
05 every 3 days (18 hr/exposure). At the end of the second
passage, cells were harvested and aliquots were analyzed
directly, cryopreserved, or used to seed 6 cm x 4 mm
knitted dacron TM grafts (CR BARD, Billerica, MA)
according to a modification of the 4-step method of Yates
10 (0.75 x 10^6 cells were added to the autologous blood
during the second and third step). Animals were
anesthetized and 6 cm segments of both carotid arteries
were replaced with the seeded grafts as described. Each
animal received an implant seeded with infected
15 endothelial cells and a contralateral graft seeded with
MOCK infected cells. Five weeks after implantation, the
animals were anesthetized and the grafts were harvested
and analyzed.

Replication-defective retroviruses with amphotropic
20 host range were used to stably introduce a reporter gene
into the genomic DNA of the endothelial cells. The lac Z
gene was used as the reporter gene because its product of
expression, beta-galactosidase, can be detected in situ
through the use of enzyme histochemical assays that stain
25 the cell's cytoplasm blue. (Example 3). Efficiency of
retroviral infection was estimated by Southern analysis
which detects proviral sequences and by the cytochemical
stain for viral-expressed beta-galactosidase which
detects infected cells in situ.
30 There were two recombinant retroviruses used in
these studies. The BAG vector has been described

-44-

previously by Price et al., Proceedings of the National
Academy of Science USA, 84: 156-160 (1987). The BAG
virus, containing the Lac Z-gene, expresses
Beta-galactosidase from the 5' LTR (Long Terminal Repeat)
05 and a selectable marker (Neo) from an SV40 derived pro-
moter which confers resistance to kanamycin in
prokaryotes and G418 in eukaryotes. Approximately 5-15%
of the endothelial cells exposed to the BAG virus were
infected (summarized in Table II); cultivation of these
10 cultures in media supplemented with the aminoglycoside
G418 effectively selected for transduced cells.

The BAL vector is derived from the previously
described BA-LDLR vector except LDLR cDNA sequences were
replaced with the coding sequences for the E. coli
15 beta-galactosidase gene. The higher titer BAL virus,
containing the Lac Z-gene, expresses beta-galactosidase
from a promoter derived from the chicken beta-actin.
Approximately 50% of the cells exposed to the BAL virus
were transduced as measured by Southern analysis and by
20 the in situ cytochemical stain for beta-galactosidase.

In Southern analysis, high molecular weight DNA was
isolated and an aliquot (10 ug) was digested with Kpn I,
fractionated on a 1% agarose gel, transferred to zetabind
and probed with a 1200 bp Cla I/ECORI fragment that had
25 been labeled to a high specific activity according to the
method of Feinberg and Volt.

Cytochemical characterization of cultured
endothelial cells was demonstrated by both phase contrast
and fluorescent micrographs. Retrovirus-infected
30 endothelial cells were analyzed at the time of seeding
and after removal from the implanted graft for uptake of

DIL-AC-LDL and for expression of viral-directed beta-galactosidase. Pre-seeded and post-implantation cells that had been inoculated with DIL-AC-LDL were assessed using phase contrast and fluorescent
05 micrographs. Pre-seeded and post-implanted cells were also analyzed for the expression of viral directed beta-galactosidase.

At the time of seeding, the cultures were analyzed for endothelial cell specific function (i.e., uptake of
10 acetylated LDL, and the presence of Von Willebrands factor) and for the presence of antigens specific for smooth muscle cells. These analyses indicated that more than 98% of the cells from each isolate were functioning endothelial cells.

15 The experimental system was a modification of a previously described dog model that has been used to study graft repopulation. Dogs 1-3 received implants that had been seeded with BAG-infected, unselected endothelial cells while dogs 4-7 initially received
20 implants seeded with the BAL-infected, unselected endothelial·cells. While these experiments were in progress, endothelial cells from dogs 4-7 were also infected with the BAG virus and expanded in culture in the presence or absence of G418, an aminoglycoside that
25 selects for BAG-transduced cells. After 5 weeks, the grafts were explanted for analysis and new grafts were implanted in dogs 4-7 (referred to as dogs 4'-7'). Each of these dogs received a graft seeded with endothelial cells that were infected with BAG virus and selected in
30 G418 and a contralateral graft seeded with BAG infected

-46-

unselected endothelial cells. The second set of grafts
were again explanted after 5 weeks.

· Table II summarizes the results of these
experiments. Animals 4' through 7' represent the second
05 experiment performed on animals 4 through 7 (see text).
BAG-U represents BAG infected endothelial cells
unselected with G418 for transduced cells. BAG-S
represents BAG infected endothelial cells selected with
G418 for transduced cells. BAL represents BAL infected
10 endothelial cells. MOCK represents MOCK infected cells.
E is an indication of the efficiency of infection as
measured by the beta-galactosidase stain. Patency of the
graft after 5 weeks is indicated with a (Y) yes or (N)
no. Coverage of the graft is an indication of the per
15 cent of the surface repopulated as measured by scanning
electron microscopy.

-47-

Table II. Summary of Implantation Experiments

Animal+   Experimental Graft   Control Graft

		Infection		Explant		Infection		Explant	
		Virus	E	Patent	Coverage	Virus	E	Patent	Coverage
05	1	BAG-U	15	Y	50-90	MOCK	O	Y	50-90
	2	BAG-U	5	Y	50-90	MOCK	O	Y	50-90
	3	BAG-U	5	Y	50-90	MOCK	O	Y	50-90
	4	BAL	40-60	N	-	MOCK	O	N	-
	5	BAL	40-60	Y	O	MOCK	O	Y	O
10	6	BAL	40-60	Y	50-90	MOCK	O	Y	50-90
	7	BAL	40-60	Y	50-90	MOCK	O	Y	50-90
	4'	BAG-S	100	Y	50-90	BAG-U	10	N	-
	5'	BAG-S	100	Y	0	BAG-U	10	N	-
	6'	BAG-S	100	Y	0	BAG-U	10	Y	50-90
15	7'	BAG-S	100	Y	50-90	BAG-U	10	Y	0

Analysis of the explanted grafts revealed that 18 of
22 remained patent after 5 weeks. Scanning electron
microscopy demonstrated a lining of cells with
endothelial-like morphology on the luminal surface of 14
of 18 patent grafts. When present, the endothelial cell
lining was incomplete. (50-90% of the total surface) and
patchy in distribution with a paucity of cells seen along
the peaks of the crimped dacron graft. A portion of each
graft was fixed, stained for cells that express
viral-derived beta-galactosidase and visualized through a
high power dissecting microscope with a magnification of
750X. Each graft seeded with infected endothelial cells
that remained patent and retained luminal cells did

-48-

contain beta-galactosidase-positive cells on the lumen of
the vessel. Contralateral grafts seeded with
MOCK-infected endothelial cells never demonstrate
positive staining cells.

05   In situ analysis of the grafts was performed for
viral-transduced cells. Longitudinal sections of the
genetically engineered implants were analyzed for cells
that express viral-expressed beta-galactosidase. Grafts
were cut longitudinally and a portion fixed in 0.5%

10 gluteraldehyde for 5 minutes, washed in PBS three times,
and incubated in x-gal solution for 2 hours and the
luminal surface photographed with a Leitz dissecting
microscope. The grafts were visualized enface and
several interesting aspects of the seeding pattern were

15 observed. The density of transduced cells was greatest .
in the deeper surfaces of the crimped graft. This was
visualized under low power as rings of blue staining
cells that line the crevices of the serrated surface and
correlates with the variation in surface endothelial cell

20 repopulation visualized by scanning electron microscopy.
In addition there was no regional variation in the
density or pattern of transduced cells with respect to
proximity to the distal or proximal anastomosis.
Finally, in each case the proportion of luminal cells

25 which were positive for beta-galactosidase seemed
qualitatively lower than the preparation of
beta-galactosidase positive cells that were seeded.
       Cells were enzymatically harvested from the luminal
surface of portions of the grafts to permit a more

30 detailed characterization. Primary cultures of cells
were established and expanded in vitro for approximately
2-3 weeks prior to analysis. Genetically modified
endothelial cells were identified in this population of

cells by Southern analysis and by the cytochemical assay
for viral-expressed beta-galactosidase. The majority of
cells harvested from the graft and expanded <u>in vitro</u>
retained differentiated endothelial function (less than
05 95%). However, the proportion of cells that expressed
viral directed beta-galactosidase or contained proviral
sequences was consistently diminished when compared to
the cultures analyzed at the time of seeding. This
disparity is due in part to the partial repopulation of
10 grafts with endogenous cells by growth through
interstices or from the anastomoses.

Industrial Utility
        This invention has industrial applicability in
improving performance (e.g., duration, patency,
15 thrombogenicity) of vascular grafts or implants, made of
synthetic materials, which are often used in treating
advanced atherosclerotic disease. It is also useful in
providing hormones, enzymes and drugs to mammals, includ-
ing humans, in need of such substances. For example, it
20 can be used to provide a continuous supply of a hormone
which otherwise would be administered on a intravenously,
intramuscularly or subcutaneously. It is particularly
valuable in providing such substances which are needed
for extended periods of time.

25 Equivalents
        Those skilled in the art will recognize, or be able
to ascertain using no more than routine experimentation,
many equivalents to the specific embodiments of the
invention described specifically herein. Such
30 equivalents are intended to be encompassed in the scope
of the following claims.

-50-

CLAIMS

1. Endothelial cells capable of expressing incorporated
    genetic material of interest.

2. Modified human endothelial cells comprising genetic
05   material of interest encoding a selected protein or
     a selected polypeptide, the genetic material of
     interest stably incorporated into the genome of the
     endothelial cells.

3. Endothelial cells of Claim 2 in which the
10   incorporated genetic material is DNA or RNA which
     does not normally occur in endothelial cells; DNA or
     RNA which normally occurs in endothelial cells but
     is not expressed in them at levels which are
     biologically significant; DNA or RNA which normally
15   occurs in endothelial cells and has been modified so
     that it is expressed in endothelial cells; and any
     DNA or RNA which can be modified to be expressed by
     endothelial cells, alone or in any combination
     thereof.

20 4. Endothelial cells of Claim 3, wherein the
     incorporated genetic material additionally comprises
     genetic material encoding at least one selectable
     marker.

5. Endothelial cells of Claim 4 in which at least one
25   selectable marker is a dominant selectable marker.

6. Endothelial cells of Claim 5 in which the dominant selectable marker is a gene encoding antibiotic resistance.

7. Human endothelial cells comprising a recombinant genome, the recombinant genome comprising incorporated DNA coding for a selected protein or a selected polypeptide.

8. Human endothelial cells of Claim 7, wherein the incorporated DNA is selected from the group consisting of DNA which does not normally occur in endothelial cells; DNA which normally occurs in endothelial cells but is not expressed in them at levels which are biologically significant; DNA which normally occurs in endothelial cells and has been modified so that is is expressed in endothelial cells; and any DNA which can be modified so that it is expressed in endothelial cells, alone or in any combination thereof.

9. Human endothelial cells of Claim 8 in which the incorporated genetic material encodes a hormone, an enzyme or a drug.

10. Human endothelial cells of Claim 9, wherein the incorporated genetic material additionally comprises genetic material which encodes at least one dominant selectable marker.

-52-

11. Cultured endothelial cells containing incorporated
    genetic material encoding a) a selected protein not
    normally made in endothelial cells or a selected
    polypeptide not normally made in endothelial cells
05 and a b) a neo gene, said neo gene being a bacterial
    gene derived from the transposon Tn5.

12. Human endothelial cells capable of expressing a
    protein or a polypeptide not normally made at
    biologically significant levels in human endothelial
10 cells.

13. Human endothelial cells expressing incorporated
    genetic material, the incorporated genetic material
    being selected from the group consisting of: a)
    genetic material encoding a hormone not normally
15 made at biologically significant levels in
    endothelial cells; b) genetic material encoding an
    enzyme not normally made at biologically significant
    levels in endothelial cells; c) genetic material
    encoding a drug not normally made at biologically
20 significant levels in endothelial cells; and d)
    genetic material encoding at least one dominant
    selectable marker.

14. Endothelial cells transduced with DNA encoding a
    protein or a polypeptide of interest.

25 15. Endothelial cells of Claim 14 wherein the DNA is
    selected from DNA which encodes a protein or a
    polypeptide which is secreted from the cells; DNA

which encodes a protein or a polypeptide which is a
membrane receptor; and DNA which encodes a protein
or a polypeptide which is an intracellular enzyme.

16. Endothelial cells of Claim 15 additionally
05   comprising DNA encoding at least one selectable
        marker.

17. Endothelial cells of Claim 16 in which the DNA
        encoding at least one selectable marker is a <u>neo</u>
        gene.

10 18. Endothelial cells of Claim 17 in which the DNA
        encodes human parathyroid hormone, low density
        lipoprotein receptor or beta-galactosidase.

19. A method of making endothelial cells which express
        incorporated genetic material of interest which
15   encodes at least one protein of interest or at least
        one polypeptide of interest, comprising the steps
        of:
        a.   contacting cultured endothelial cells with
        media containing an infectious recombinant
20   retrovirus having a recombinant genome comprised of
        the genetic material of interest; and
        b.   maintaining the cultured endothelial cells and
        the media containing the infectious recombinant
        retrovirus under conditions appropriate for
25   infection of the endothelial cells by recombinant
        retrovirus.

-54-

20.   A method of Claim 16 in which the genetic material
      of interest encodes a hormone, an enzyme or a drug
      not normally expressed in endothelial cells.

21.   A method of Claim 20 in which the recombinant genome
05   of the retrovirus additionally comprises genetic
      material encoding at least one dominant selectable
      marker.

22.   A method of making endothelial cells, capable of
      expressing incorporated genetic material encoding at
10   least one protein or at least one polypeptide of
      interest, comprising the steps of:
      a.   transducing cultured endothelial cells with
      genetic material encoding at least one protein
      or at least one polypeptide of interest and
15   b.   culturing the transduced endothelial cells
      under conditions appropriate for their growth.

23.   A method of providing a hormone, an enzyme or a drug
      to the body, comprising the steps of:
      a.   transducing endothelial cells with genetic
20   material encoding the hormone, the enzyme or the
      drug and
      b.   introducing the transduced endothelial cells
      into the body or applying the transduced endothelial
      cells to a surface of the body.

25 24.   A method of providing a hormone, an enzyme or a
      drug to the body, comprising the steps of:
      a.   infecting endothelial cells with a

recombinant retrovirus, the recombinant
retrovirus having a recombinant genome comprised
of:

1)   genetic material encoding the hormone,
05   the enzyme or the drug;

2)   the long terminal repeat sequences,
        the tRNA binding site and the Psi
        packaging site derived from an
        amphotropic retrovirus; and

10   3)   at least one promoter of eukaryotic
                origin; and

b.   introducing the resulting endothelial cells
into the body or applying the resulting endothelial
cells to the surface of the body.

15 25.   A method of Claim 24 in which the recombinant genome
          is additionally comprised of a promoter of eukary-
          otic origin which can be modulated by an external
          cue.

    26.   A method of Claim 25 in which the recombinant genome
20   is additionally comprised of genetic material
          encoding at least one dominant selectable marker.

    27.   A method of Claim 24 in which the genetic material
          encoding at least one dominant selectable marker is
          a gene encoding antibiotic resistance or a gene that
25   complements a genetic defect in the host species.

    28.   A method of reducing the thrombogenicity of a
          synthetic prosthetic vessel, comprising applying to

-56-

the vessel endothelial cells transduced with genetic
material encoding a thrombolytic protein or a
portion thereof.

29.   A method of Claim 28 wherein the genetic material
05   encodes tissue plasminogen activator or
      streptokinase.

30.   A method of producing a confluent layer of
      endothelial cells lining the inner surface of a
      prosthetic vessel, comprising applying to the inner
10   surface of the vessel endothelial cells comprising
      incorporated genetic material encoding an
      endothelial cell mitogen, under conditions
      appropriate for endothelial cell proliferation.

31.   A method of Claim 30, wherein the genetic material
15   encodes endothelial cell growth factor.

32.   A method of producing a prosthetic vessel having
      endothelial cells which express incorporated genetic
      material of interest lining the inner surface of the
      vessel, comprising seeding the inner surface of the
20   vessel at subconfluent levels with endothelial cells
      produced by the method of Claim 19.

33.   A method of producing a prosthetic vessel lined with
      endothelial cells which express incorporated genetic
      material of interest, comprising the steps of:

a.   contacting cultured endothelial cells with
media containing an infectious recombinant
retrovirus having a recombinant genome comprised of
the genetic material of interest;

05   b.   maintaining the cultured endothelial cells with
media containing an infectious recombinant
retrovirus, under conditions appropriate for
infection of the endothelial cells by recombinant
retrovirus; and

10   c.   seeding the endothelial cells infected in (b)
onto the inner surface of a prosthetic vessel at
subconfluent densities, under conditions appropriate
for maintenance of the endothelial cells.

34.   A method of implanting a prosthetic vessel lined
15   with endothelial cells which express incorporated
genetic material of interest in a mammal, comprising
the steps of:
a.   contacting cultured endothelial cells with
media containing an infectious recombinant
20   retrovirus having a recombinant genome comprised of
the genetic material of interest;
b.   maintaining the cultured endothelial cells with
media containing an infectious recombinant
retrovirus under conditions appropriate for
25   infection of the endothelial cells by recombinant
retrovirus;
c.   seeding the endothelial cells infected in (b)
onto the inner surface of a prosthetic vessel at
subconfluent densities under conditions

-58-

appropriate for maintenance of the endothelial
cells; and

d.   introducing the prosthetic vessel formed in (c)
into the mammal.

05 35.   A method of enhancing binding of endothelial cells
to a surface of a prosthetic vessel, comprising the
steps of:

a.   applying to the surface of the vessel a ligand,
to produce a ligand-bearing surface;

10   b.   seeding onto the ligand-bearing surface
endothelial cells comprising incorporated genetic
material encoding a membrane receptor which binds
the ligand; and

c.   maintaining the vessel under conditions

15   appropriate for binding of ligand and membrane
receptor.

36.   A method of inhibiting growth of smooth muscle cells
in a synthetic vascular implant, comprising applying
to the implant endothelial cells comprising

20   incorporated genetic material encoding a product
which inhibits smooth muscle cell growth.

	LTR		▤	GAG	POL	ENV	LTR	

5'ss   AUGgag   3'ss
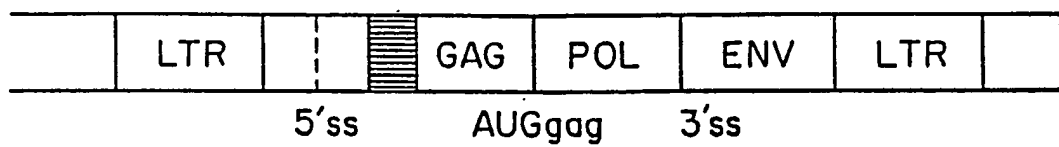
Fig. 1

Fig 2a   pLJ
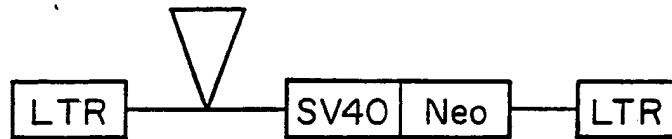
Fig 2b   pEm
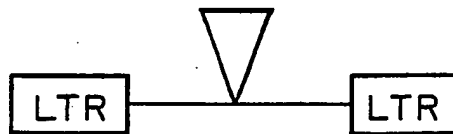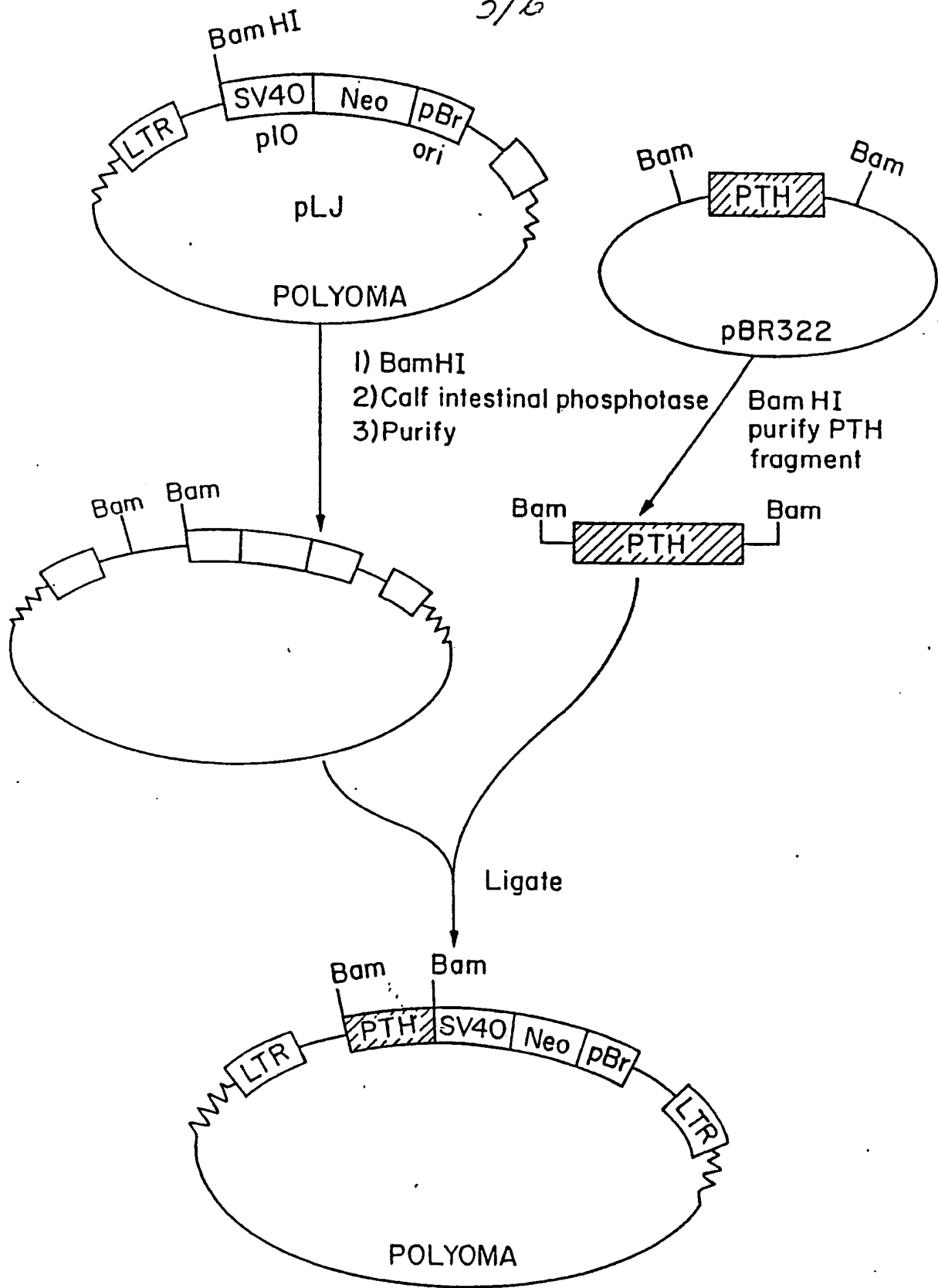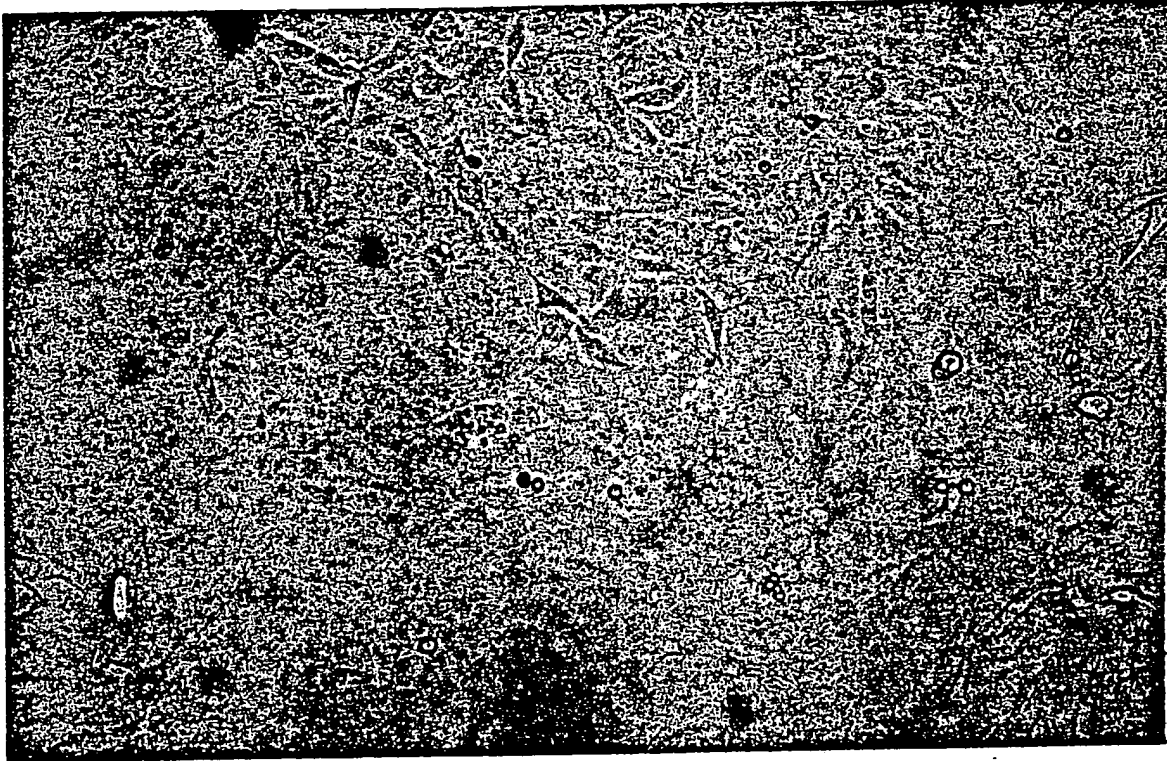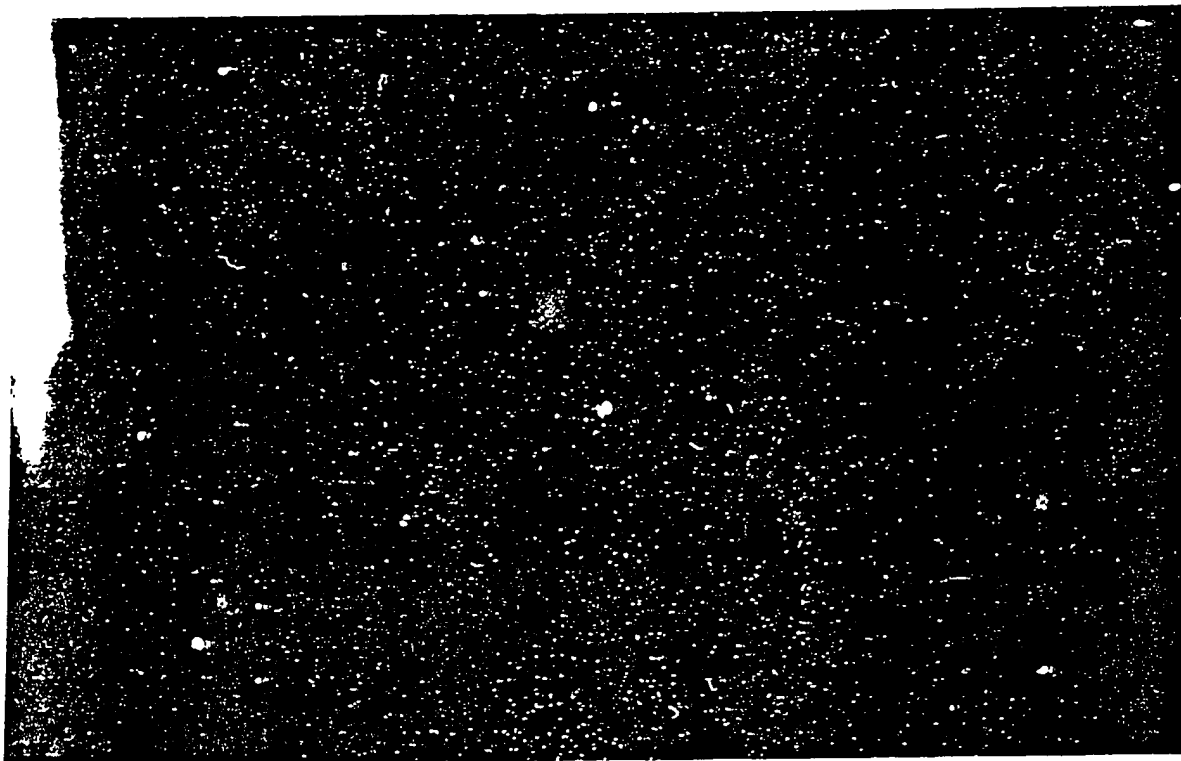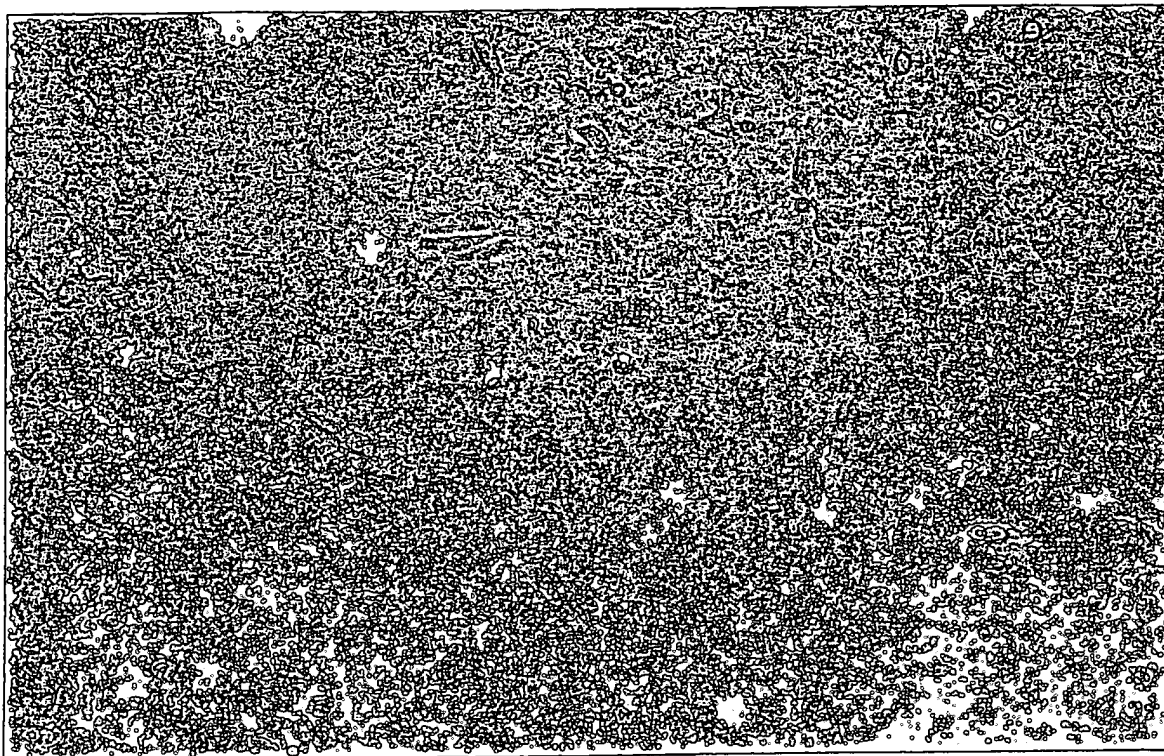
SUBSTITUTE SHEET

Fig. 3

FIG.4A

FIG.4B

FIG.4C

FIG.4D
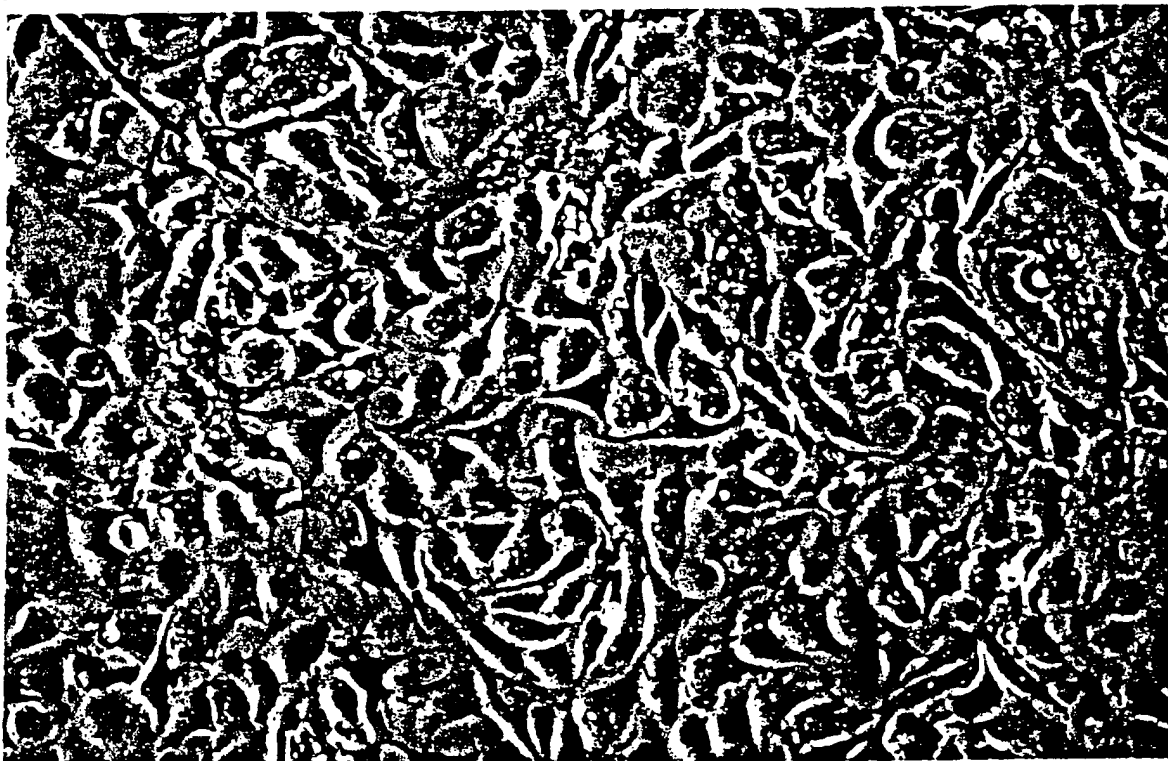
FIG.5A
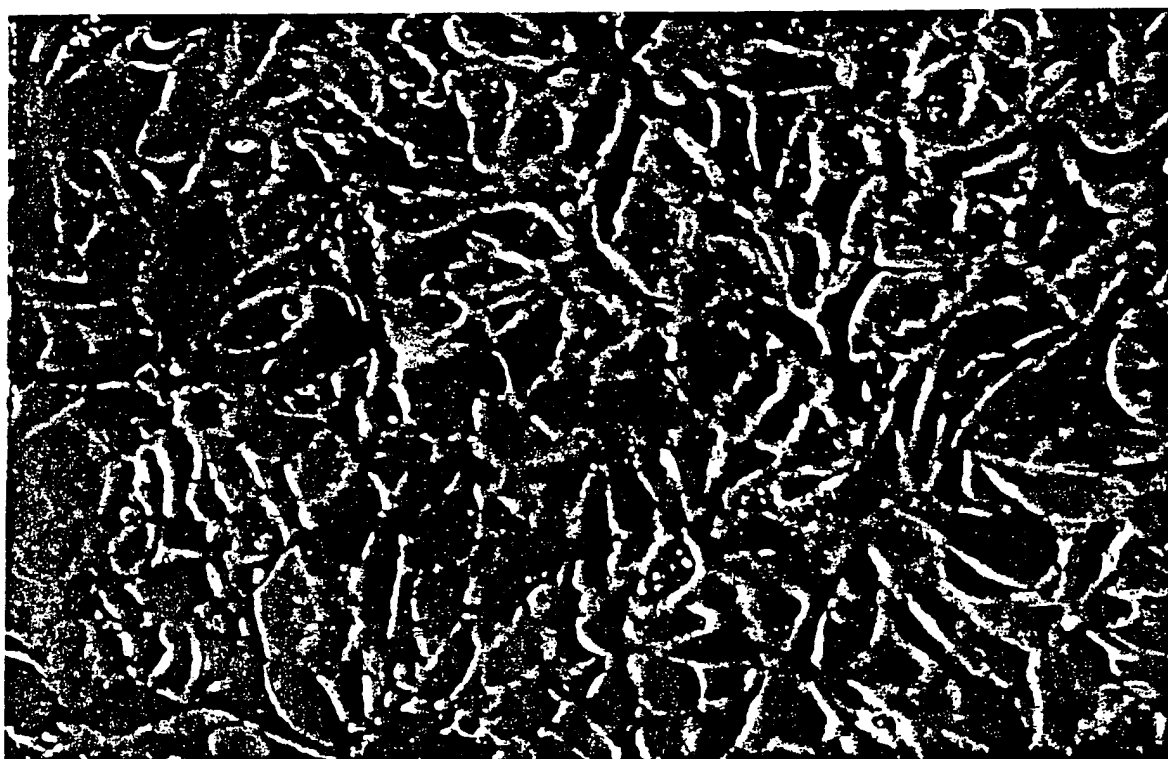
FIG.5B

INTERNATIONAL SEARCH REPORT

International Application No   PCT/US 88/04383

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4:   C 12 N 5/00; C 12 N 15/00; A 61 L 27/00; A 61 L 33/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	WO, A, 87/00201 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 15 January 1987 see claims	1-22
A	Proceedings of the National Academy of Sciences of the USA, volume 81, no. 20, October 1984, (Washington, US), R.D. Cone et al.: "High-efficiency gene transfer into mammalian cells: Generation of helper-free recombinant retrovirus with broad mammalian host range", pages 6349-6353 see the whole article	19-21
A	Chemical Abstracts, volume 110, 1988, (Columbus, Ohio, US), D.L. Mooradian: "Transforming growth factor production by Rous sarcoma virus-transformed rat cerebral endothelial cells", see abstract 21757g, & Diss. Abstr. Int. B 1988, 49(4), 1099 ./.	1-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd March 1989	2 0. 04. 89

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	DNA, volume 7, no. 9, 1988, Mary Ann Liebert, Inc., Publishers, R.W. Connors et al.: "DHFR co-amplification of t-PA in DHFR bovine endothelial cells: In vitro characterization of the purified serine protease", pages 651-661 see the abstract	1,14-17
T	Science, volume 243, 13 January 1989, J.A. Zwiebel et al.: "High-level recombinant gene expression in rabbit endothelial cells trans- duced by retroviral vectors", pages 220-222 see the whole article	
A	EP, A, 0206025 (THOMAS JEFFERSON UNIVERSITY) 30 December 1986 see page 13, line 8 - page 17, line 31	30,32-34

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☒ Claim numbers OO).... because they relate to subject matter not required to be searched by this Authority, namely:

    OO) 23-27,34   See PCT Rule 39.1(iv)   Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2.☐ Claim numbers..........., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers........... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple Inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 12/04/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8700201	15-01-87	AU-A-	6131086	30-01-87
		EP-A-	0228458	15-07-87
		JP-T-	63500492	25-02-88
EP-A- 0206025	30-12-86	AU-A-	5830586	11-12-86
		JP-A-	62049857	04-03-87

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82